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Cyclosporine A-induced increase in glomerular cyclic GMP in rats and the involvement of the endothelin$_B$ receptor

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1 A transient two fold increase in the cyclic GMP content was observed in rat freshly isolated glomeruli 6 to 9 h after a single subcutaneous injection of 20 mg kg^{-1} cyclosporine A (CsA) in conscious animals.

2 *In vitro* stimulation with endothelin 3 (ET-3) of isolated glomeruli obtained from CsA-untreated rats resulted in a dose-dependent increase in cyclic GMP content. The increase observed with 10 nM ET-3 was similar to that observed in glomeruli isolated 9 h after *in vivo* CsA administration.

3 The rise in glomerular cyclic GMP content after *in vivo* CsA injection was prevented by *in vivo* treatment with L-NAME (10 mg kg^{-1}) or by *in vitro* calcium deprivation of the incubation medium.

4 The stimulating effects of CsA on glomerular cyclic GMP content were inhibited by *in vivo* administration of the ET_B receptor antagonist BQ-788 (2 mg kg^{-1}) but not by the ET_A receptor antagonist BQ-123 (2 mg kg^{-1}).

5 The maximum increase in glomerular cyclic GMP content induced *in vitro* by acetylcholine (100 μM) and by ET-3 (100 nM) was slightly lower (approximately by 20–25%, $P<0.05$) in glomeruli from CsA-treated rats than in glomeruli from untreated rats. In contrast, the maximum increase achieved with 1 μM sodium nitroprusside was similar in both groups.

6 A single subcutaneous injection of CsA did not significantly alter the glomerular mRNA expression of constitutive endothelial NO synthase (eNOS), as evaluated by RT–PCR, whereas the mRNA expression of the inducible NO synthase (iNOS), which follows pretreatment with lipopolysaccharide, was prevented.

7 These results indicate that *in vivo* administration of a single dose of cyclosporine A transiently increases the cyclic GMP content of freshly isolated glomeruli, and that activation of ET_B receptors and stimulation of the NO pathway are involved in this process. Furthermore, a single administration of CsA does not impair eNOS mRNA expression and only slightly reduces NO-dependent glomerular cyclic GMP production.

Keywords: Cyclosporine A; endothelins; nitric oxide; nitric oxide synthases; cyclic GMP; kidney; glomerulus

Introduction

An increased synthesis of endothelins is one of the major responses to exposure to deleterious conditions in several cell types including renal endothelial, mesangial and epithelial cells (Sakamoto *et al*., 1990; Marsden *et al*., 1991; Kohan, 1991). The main effect of this increase is a potent local vascular constriction. Paradoxically, endothelins may also induce a dose-dependent vasodilatation which results from an activation of the nitric oxide (NO) pathway. Several experiments have revealed this surprising finding: (i) in the isolated perfused mesentery of the rat the endothelin-induced dilatation is abolished by both removal of the endothelial cells and by methylene blue, an inhibitor of the soluble guanylate cyclase (Warner *et al*., 1989); (ii) incubation of rat isolated glomeruli with endothelins elicits a dose-dependent increase in glomerular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) content which also results from an activation of the NO pathway because it is suppressed by both methylene blue and N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthases (Edwards *et al*., 1992; Owada *et al*., 1994); and, (iii) in the rabbit perfused kidney, a selective ET_B receptor antagonist potentiates the increase in perfusion pressure (which depends on the vascular tone) induced by endothelin-1 (ET-1) (D'Orléans-Juste *et al*., 1994). However, this mechanism has been observed exclusively during *in vitro* pharmacological endothelin receptor stimulation, and there is no evidence yet that it is involved in pathological and/or therapeutic *in vivo* conditions.

With regard to the glomerulus, the therapeutic use of cyclosporine A (CsA) is detrimental and involves the endothelin pathway. Previous studies have demonstrated that CsA has the ability to induce the synthesis of endothelins in cultured endothelial and mesangial cells (Bunchman & Brookshire, 1991; Takeda *et al*., 1992) and to increase endothelin concentration in blood and urine (Kon *et al*., 1990; Perico *et al*., 1992). This increased endothelin synthesis seems indeed to be largely responsible for the potent constrictive effect of CsA on intrarenal vessels. The most convincing results which support this assertion are that the selective blockade of ET_A receptors is able to prevent almost completely the CsA-induced renal haemodynamic alterations in anaesthetized rats (Fogo *et al*., 1992; Kivlighn *et al*., 1994). There is thus general agreement that endothelin synthesis is a key-mechanism in the renal haemodynamic toxicity of CsA.

However, the chain of events from CsA to renal haemodynamic alterations is found to be less simple when the innermost mechanisms are examined, mainly because of two considerations. (1) There are two different kinds of endothelin receptors. ET_A receptors are mainly expressed on smooth muscle cells and are almost solely involved in contraction. ET_B receptors are predominantly located on endothelial cells and may be involved either in vascular constriction or in vascular dilatation depending on the species and the vascular territory

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(see Haynes, 1955 for review). (2) It is therefore conceivable that the apparent constrictive effects of CsA on intrarenal vessels via increased endothelin synthesis do not merely involve an exclusive mechanism. On the contrary, they may result from the summation of concomitant and opposing effects: ET_A receptor-mediated constriction and ET_B receptor-induced activation of the NO-pathway. This study was therefore designed to test this hypothesis and to examine further whether CsA, ET_B receptors and the NO pathway are somewhat connected and involved in the final effects of CsA on the glomerulus. For this purpose, the acute effects of *in vivo* cyclosporine administration on glomerular cyclic GMP content and on the expression of the constitutive and inducible NO synthase mRNAs were assessed.

Methods

Animals and in vivo *experimental protocols*

Male Sprague-Dawley rats (180–250 g) purchased from IFFA CREDO farm, were used. All surgical procedures were carried out with standard guidelines, for the care and use of animals for experimental purposes. CsA-treated rats were submitted to a single subcutaneous injection of 20 mg kg^{-1} cyclosporine diluted in olive oil. It has been previously demonstrated that this dose induces renal haemodynamic alterations without any effect on systemic blood pressure (Murray *et al*., 1985). Control rats received an equivalent volume of olive oil only. In pilot experiments, blood was withdrawn after aortic cannulation to measure the whole blood cyclosporine level (RIA, Cyclo-trac SP Kit, Incstar Co, Stillwater, Minnesota, U.S.A.) 1, 6, 9, 18 and 36 h after CsA injection. Kidneys were removed for isolation of glomeruli. The final experiments included 7 different groups of rats. Group 1: control; group 2: CsA-treated rats killed 9 h after CsA administration; group 3: same as group 2 with additional subcutaneous administration of 10 mg kg^{-1} N^{ω}-nitro-L-arginine methyl ester (L-NAME, an inhibitor of NO synthases) 2 h before death; group 4: same as group 2 with additional subcutaneous administration of 2 mg kg^{-1} BQ-123 (ET_A receptor antagonist) 2 h before death; group 5: same as group 2 with additional subcutaneous administration of 2 mg kg^{-1} BQ-788 (ET_B receptor antagonist) 2 h before death; group 6: lipopolysaccharide (LPS)-treated rats (jugular vein i.v. administration of a 50 μg bolus of lipopolysaccharide six hours before the kidneys were removed); group 7: CsA and LPS-treated rats.

Isolation of rat glomeruli

The animals were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.) and the abdominal aorta was cannulated in order to rinse the kidney *in situ* by infusing 50 ml of cold (5°C) HEPES buffer. The kidneys were then removed and the cortexes were dissected out manually. Glomeruli were isolated by graded sieving as previously described (Bascands *et al*., 1989). In our experimental conditions, we obtained about 15,000 glomeruli per kidney. Isolated glomeruli were resuspended in HEPES buffer to reach a final concentration of about 1000 glomeruli per incubation. The HEPES buffer composition was as follows (mM): NaCl 137, KCl 5, $CaCl_2$ 1, NaH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5.6, sodium acetate 10, DL-lactate and N-2-hydroxyethylpiperazine-N1-2-ethanesulphonic acid (HEPES) 20, pH 7.4.

Induction of intracellular cyclic GMP accumulation in isolated glomeruli

To evaluate baseline cyclic GMP content after the various *in vivo* treatments, glomeruli were incubated with the phosphodiesterases inhibitor 3-isobutyl-1-methyl xanthine (IBMX) at 0.5 mM final concentration for five minutes immediately after isolation. In fact, when glomeruli were incubated at 37°C, the baseline glomerular cyclic GMP content remained stable for at least one hour after glomerulus isolation. For *ex vivo* pharmacological experiments, glomeruli were preincubated for 30 min at 37°C in the presence or absence of 0.1 mM N^G-monomethyl-L-arginine (L-NMMA). Then a 5 min stimulation period was started on addition of the appropriate concentration of ET-3, acetylcholine or calcium ionophore A 23187. All these drugs were previously diluted in HEPES buffer containing IBMX (0.5 mM final concentration). The incubation was stopped by adding 2 ml of methanol/formic acid solution (95/5, v/v) to each tube. The samples were stored frozen until cyclic GMP measurement. Each experiment in the various conditions was performed in triplicate.

Measurement of intracellular cyclic GMP content in isolated glomeruli

Frozen glomerular samples were thawed, sonicated (Branson sonifier 250) and centrifuged (10 min at $4000 \times g$). The pellets were discarded and the supernatants were concentrated to dryness at room temperature with a speed vack concentrator (Savant Instruments, U.S.A.). Dried samples were reconstituted in phosphate buffer. Concentrations of cyclic GMP were determined after acetylation of the supernatants with the EIA kit from Cayman Chemical (Ann Arbor, MI, U.S.A.) according to the manufacturer's instructions. Our experimental conditions gave a detection limit of 3 pmol ml^{-1} with 5 ± 2 and 6 ± 2% intrassay and interassay variations, respectively. Results are expressed as fmol cyclic GMP generated μg^{-1} protein. In each experiment, samples of renal glomerulus suspension were used to determine the protein content. After solubilization for 15 min at 100°C with 1M NaOH, proteins were measured by the method of Lowry *et al*. (1951) with serum albumin as standard.

Analysis of eNOS and iNOS mRNA by reverse transcriptase polymerase chain reaction (RT–PCR)

Total RNA was extracted from frozen glomeruli according to the method described by Chomczinski & Sacchi (1987). RNA pellets were dried and dissolved in water containing diethyl pyrocarbonate (0.1%). The RNA was assayed by u.v. spectrophotometry at 260 and 280 nm. Only RNA preparations with an OD260/OD280 ratio between 1.9 and 2.1 were used for cDNA synthesis.

First strand cDNA was synthesized with 0.75 μg of total RNA, oligonucleotides ($pd(T)_{15}$) for priming, RNase inhibitor (32 units), DTT (25 mM), dNTPs (5 mM), 5X RT buffer and reverse transcriptase (M-MLV- from Gibco BRL: 200 units). After RNA denaturation (10 min, 70°C), the reaction was carried out at 42°C for 50 min with a Perkin Elmer TC-480 DNA Thermal Cycler, heated to 95°C for 5 min and chilled on ice.

For polymerase chain reaction (PCR), 10 μl of each cDNA preparation were amplified. PCR amplification was performed by adding 140 pM of each primer and 2 u Taq polymerase (Appligene) to a final volume of 100 μl PCR buffer. For iNOS mRNA amplification, samples were denatured for 3 min at 94°C; then PCR was performed for 35 cycles (1 min at 94°C, 1 min at 63°C, 1 min at 70°C) followed by 10 min at 70°C. For eNOS mRNA amplification, samples were denatured for 3 min at 94°C; then PCR was performed for 35 cycles (1 min at 94°C, 1 min at 52°C, 1.30 min at 72°C) followed by 10 min at 72°C. The PCR products for iNOS and eNOS were amplified from the same cDNA and then electrophoresed.

Amplification in the absence of cDNA did not yield any bands other than those of the primers for iNOS and eNOS mRNAs at the bottom of the gel (not shown).

The inducible NO synthase primers (Nunokawa *et al*., 1993), whose sequences corresponded to bases 1–24: 5′-ATGGCTTGCCCCTGGAAGTTTCTC-3′ (sense) and to

bases 802–827: 5′-CCTCTGATGGTGCCATCGGGCAT-CTG-3′ (antisense), were used. For the endothelial NO synthase, primers were: 5′-TACGGAGCAGCAAATCCAC-3′ for the sense primer, and the bases: 5′-CAGGCTGCAG-TCCTTTGATC-3′ for the antisense primer (Ujiie *et al*., 1994). The expected size of the PCR product was predicted to be 830 base pairs (bp) in length for iNOS and 819 bp for eNOS.

In order to check that the amplified fragments really corresponded to authentic iNOS and eNOS transcripts, we hybridized Southern blots of electrophoresed PCR products with the c-oligonucleotides located inside the synthesized cDNA sequences. The c-oligonucleotides for iNOS and eNOS were: 5′-AGCTCACTCTGGATGAGC-3′ and 5′-CTGGAACAATTTCCATCCG-3′, respectively. After amplification, 40 μl of each PCR product was electrophoresed through a 1.8–2% agarose gel, and then the gel was denatured, neutralized and blotted overnight onto a standard nylon membrane (Hybond-N-Amersham) with 20× SCC (sodium chloride/sodium Citrate) as the transfer buffer. The DNA was fixed to the nylon under u.v. radiation (254 nm) in an u.v.-crosslinker (Stratagene). Radiolabelled probe (^{32}P) was end-labelled with [γ^{32}P]-ATP (4500 Ci ml^{-1}) by use of T4 polynucleotide-kinase (Promega). After hybridization in stringent conditions with the ^{32}P probe (42°C overnight), the nylon was washed with SCC 2×/0.1% SDS once at room temperature and twice at 65°C for 30 min. Autoradiography was performed for 4 h at −80°C by use of Amersham hyperfilm-MP film with intensifying screens.

Reagents

L-NAME, L-NMMA, acetylcholine, endothelin 3 (ET-3) and calcium ionophore A23187 were purchased from Sigma Chimie (Meudon, France). BQ-788 (cis-2,6-dimethylpiperidinocarbonyl) an ET_B selective antagonist, was obtained from Neosystem Laboratoire (Strasbourg, France). BQ-123 (cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu)), a selective ET_A antagonist was a gift from the Institut H. Beaufour (Les Ullis, France). Cyclosporine A was a gift from Sandoz Laboratoires (Rueil-Malmaison, France).

Statistical analysis

Results are expressed as means±s.e. The non-parametric Mann-Whitney U test was used for comparisons between two unpaired variables. Multiple means were compared with single factor analysis of variance (ANOVA). Differences were considered significant at $P<0.05$.

Results

Time course and dose-dependent effect of treatment with CsA

The effects of *in vivo* treatment of rats with a single administration of CsA on the time courses of glomerular cyclic GMP content and of blood CsA concentration are shown in Figure 1. The glomerular cyclic GMP content peaked 9 h after CsA administration. Then it decreased to control levels after 18 h. Interestingly, the cyclic GMP content exhibited a pattern parallel to that of blood CsA level. Consequently, the following experiments were conducted on isolated glomeruli obtained 9 h after subcutaneous injection of CsA. In these conditions, the baseline glomerular cyclic GMP content of CsA-treated rats was higher than that of untreated rats (2.8±0.6 vs 5.5±0.6 fmol μg^{-1} protein, $P<0.05$). In another set of experiments, we observed an ET-3-induced dose-dependent increase in glomerular cyclic GMP level from CsA-untreated rats (Figure 2) with an ED_{50} of 50 nM. The increase observed in glomeruli from CsA-treated rats was equivalent to the stimulating effect of 10 nM ET-3 on isolated glomeruli from CsA-untreated rats. Furthermore, *in vitro* incubation of glomeruli from CsA-untreated rats with 0.5 mM CsA (a concentration similar to the maximum CsA blood level observed in our study) for 30 min elicited no change in glomerular cyclic GMP content (controls: 2.5±0.5 fmol μg^{-1} protein; +CsA for 30 min: 2.8±0.6 fmol μg^{-1} protein, $n=4$).

Relationships between the effects of CsA on glomerular cyclic GMP content and NO pathway

A single subcutaneous administration of L-NAME, 10 mg kg^{-1} two hours before glomerulus isolation decreased baseline glomerular cyclic GMP content by 45% in CsA-untreated rats and completely abolished the stimulating effect of CsA (Figure 3). Furthermore, the stimulation effect of 10 nM ET-3 on glomerular cyclic GMP content was abolished in both groups (not shown).

Calcium-dependence of glomerular cyclic GMP content

The increased glomerular cyclic GMP content in CsA-treated rats was no longer detectable when glomeruli were incubated for 30 min in a calcium-deprived medium (0.1 μM Ca^{2+}) (Figure 4). In the same conditions, the stimulating effect of 0.1 μM ET-3 was also abolished. Moreover, 1 μM of the calcium ionophore A23187 increased the glomerular cyclic GMP content of CsA-untreated and -treated rats to a similar level. Since the basal level of glomerular cyclic GMP was previously

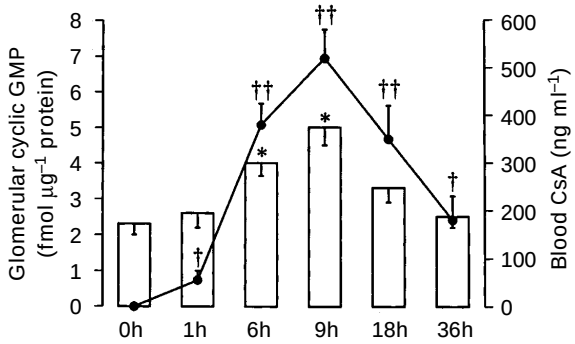

Figure 1 Changes in the blood concentration of cyclosporine A (CsA) (●) and in the cyclic GMP content (open columns) of isolated glomeruli obtained from rats at various times after a single subcutaneous dose of CsA. The glomerular cyclic GMP content is expressed as fmol μg^{-1} protein. Results are means±s.e. (vertical lines) for 3 or 4 distinct experiments; *$P<0.05$ versus cyclic GMP content at time 0; †$P<0.05$ and ††$P<0.01$ versus CsA blood concentration at time 0.

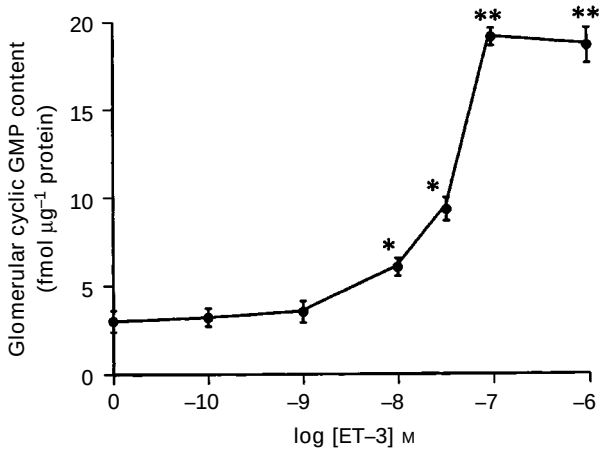

Figure 2 Dose-dependent effect of endothelin 3 (ET-3) on the cyclic GMP content of isolated glomeruli obtained from CsA-untreated rats. Results are means of at least 4 distinct experiments; vertical lines show s.e. *$P<0.05$ and ††$P<0.01$ versus non-stimulated value.

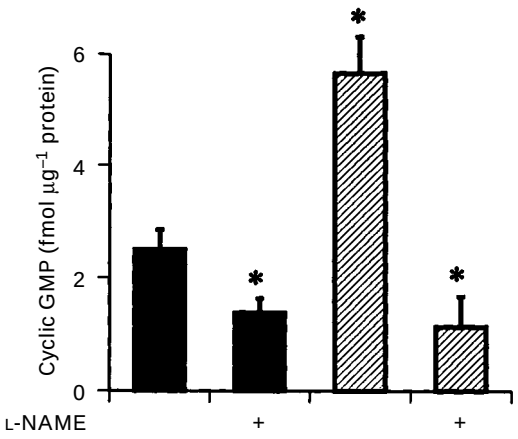

Figure 3 Effects of *in vitro* administration of L-NAME (10 mg kg^{-1}) on baseline cyclic GMP content of isolated glomeruli obtained from CsA-untreated (solid columns) and CsA-treated rats (hatched columns). Results are means ± s.e. of 6 distinct experiments. $*P<0.05$ when compared to values obtained in glomeruli from CsA-untreated rats without L-NAME.

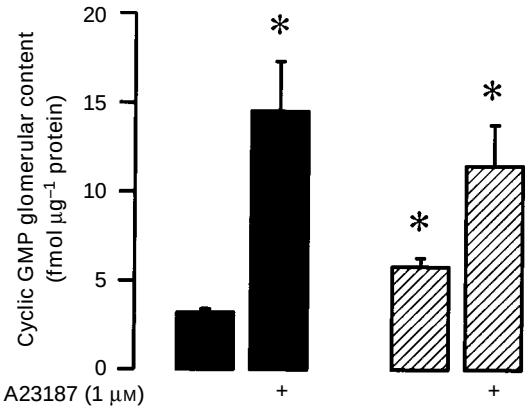

Figure 5 Effects of ionomycin (A23187) on cyclic GMP content in isolated glomeruli from CsA-untreated (solid columns) and CsA-treated rats (hatched columns). Results are absolute values expressed as means ± s.e. of at least 3 distinct experiments. $*P<0.05$ when compared to values in glomeruli from untreated rats in the absence of ionomycin.

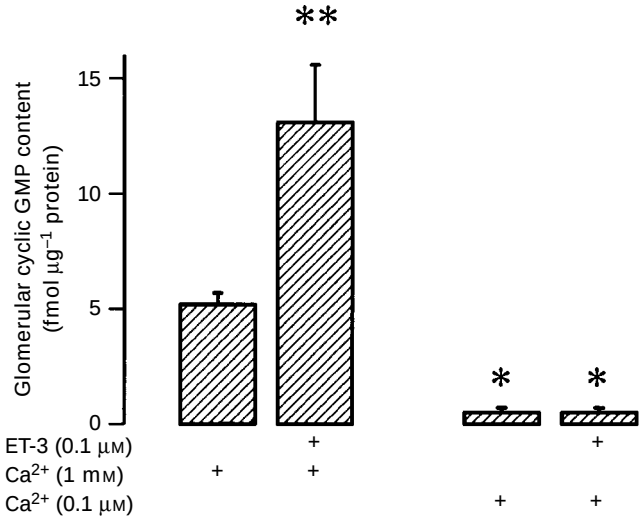

Figure 4 Effects of two different calcium concentrations in the incubation medium on the cyclic GMP level of isolated glomeruli from CsA-treated rats in the presence or absence of ET-3 (100 nM). Results are absolute values expressed as means ± s.e. of at least 3 distinct experiments. $*P<0.05$ when compared to baseline values obtained with 1 mM calcium-HEPES buffer and no stimulation with ET-3.

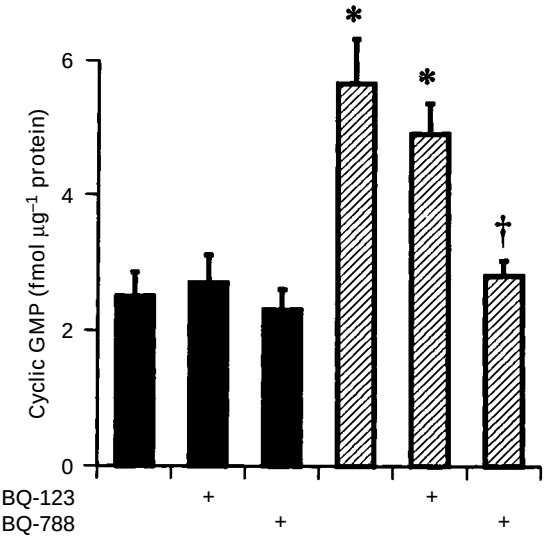

Figure 6 Effects of *in vivo* administration (2 mg kg^{-1} subcutaneously) of the two endothelin antagonists BQ-123 and BQ-788 (ET_A and ET_B antagonists, respectively) on baseline glomerular cyclic GMP content measured immediately after glomerular isolation from untreated rats (solid columns) and from cyclosporine A-treated rats (hatched columns). Results are expressed as means ± s.e. of 6 distinct experiments. $*P<0.05$ when compared to baseline values obtained in glomeruli from CsA-untreated rats. $\dagger P<0.05$ when compared with baseline value of CsA-treated rats.

increased in CsA-treated rats, the net effect of A23178 was weaker on glomeruli from CsA-treated rats than on glomeruli from CsA-untreated rats (Figure 5).

Relationships between the effects of CsA on glomerular cyclic GMP content and ET receptor subtypes

To examine the possible involvement of endothelin receptor activation, *in vivo* administration (2 mg kg^{-1}, subcutaneously, 2 h before glomerular isolation) of BQ-123 or BQ-788, respectively selective ET_A and ET_B receptor antagonists, was performed (Figure 6). Neither antagonist had any effect on baseline glomerular cyclic GMP content in CsA-untreated rats. In contrast, *in vivo* administration of BQ-788 prevented the increase in glomerular cyclic GMP content in CsA-treated rats, whereas BQ-123 had no effect (Figure 4). Preliminary *in vivo* administration of BQ-788 also prevented the *in vitro* stimulating effect of 10 nM ET-3 on glomerular cyclic GMP content (data not shown).

Effects of ET-3, ACh and SNP on glomerular cyclic GMP content

As shown in Figure 7, incubation of glomeruli from CsA-untreated rats with 100 nM ET-3 or 0.1 mM acetylcholine (ACh) induced a 5.5 fold increase in glomerular cyclic GMP content, and sodium nitroprusside (SNP) induced a 7 fold increase ($P<0.01$ versus unstimulated value). Incubation of freshly isolated glomeruli with L-NMMA (0.1 mM) reduced the baseline glomerular cyclic GMP content of CsA-treated and CsA-untreated rats to similar values (1.2 ± 0.3 fmol μg^{-1} protein and 1.4 ± 0.5 fmol μg^{-1} protein, respectively) and completely abolished the stimulating effects of both ET-3 and ACh, whereas it had no effect on the stimulating effect of sodium nitroprusside (SNP) (not shown). The concentrations of ET-3, ACh and SNP used in these experiments elicited the

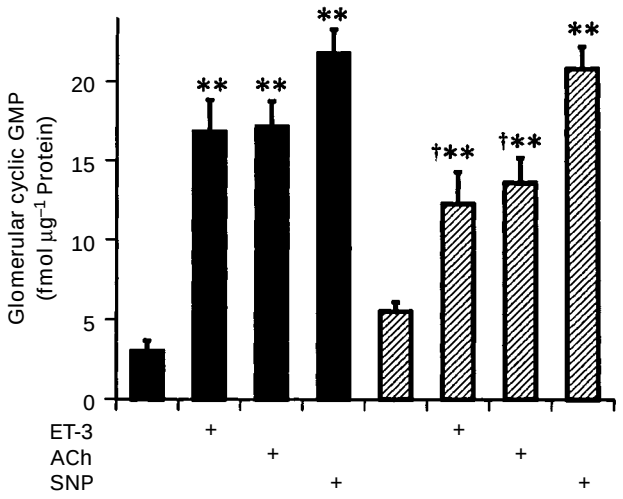

Figure 7 Effects of *in vitro* stimulation with ET-3, ACh and SNP on cyclic GMP content of isolated glomeruli obtained from CsA-untreated (solid columns) and CsA-treated rats (hatched columns). Glomeruli were stimulated with 100 nM ET-3, 0.1 mM ACh or 1 μM SNP. Results are means $\pm$ s.e. of 4 to 6 distinct experiments. $*P<0.05$ and $**P<0.01$ when compared to values obtained in glomeruli from CsA-untreated rats stimulated with neither ET-3, ACh nor SNP. $\dagger P<0.05$ when compared to values obtained in CsA untreated rats with the same stimulus.

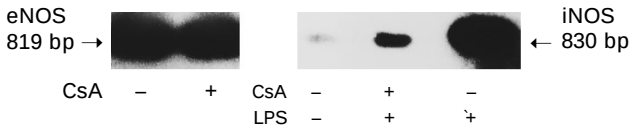

Figure 8 Southern blot analysis of eNOS (left panel) and iNOS (right panel) mRNAs from whole glomeruli after reverse transcription polymerase chain reaction (RT–PCR), transfer and hybridization with ^{32}P-labelled probes. CsA: cyclosporine A-treated rats; LPS; lipopolysaccharide-treated rats.

highest increase in glomerular cyclic GMP content. In glomeruli from CsA-treated rats, the maximum stimulating effect of ET-3 on cyclic GMP content was not additive with the effect of preliminary treatment with CsA. In addition, the effects of ET-3 and ACh on cyclic GMP production were slightly but significantly weaker in glomeruli from CsA-treated rats than in glomeruli from CsA-untreated rats. In contrast, the stimulating effect of SNP was similar in both groups (Figure 7).

Effect of CsA on constitutive and inducible nitric oxide synthase mRNAs

The mRNA of the constitutive endothelial NO synthase (eNOS) was equally present in the glomeruli from CsA-untreated and -treated rats (Figure 8). Furthermore, the mRNA of the inducible NOS was detected only at low levels in the freshly isolated glomeruli from untreated rats. When rats were pretreated with LPS to induce iNOS expression, CsA treatment completely prevented the induction of iNOS expression (Figure 8).

Discussion

The results of this study demonstrate that administration of a single subcutaneous dose of cyclosporine A to normal rats elicits a transient increase in glomerular cyclic GMP content which involves the NO-dependent pathway via activation of subtype B endothelin receptors. Our observations provide new information on two points: (1) this is the first evidence that a pharmacological *in vivo* manoeuvre relevant to clinical conditions results in activation of the NO pathway mediated through ET_B receptor activation; and (2) acute cyclosporine A administration does not reduce NO dependent cyclic GMP content in the glomerulus but rather limits its increase when the consitutive NO pathway is stimulated.

The first point of interest is that *in vivo* administration of cyclosporine A transiently increases cyclic GMP content in *ex vivo* isolated glomeruli through an endothelin-induced stimulation of the NO pathway. This increase in glomerular cyclic GMP content is NO-dependent because it was completely abolished by *in vivo* administration of L-NAME, an inhibitor of NO synthases. Furthermore, it is likely that this activation of the NO pathway depends on a constitutive calcium-dependent NO synthase because: (i) incubation of glomeruli in a calcium-deprived medium also completely abolished the increase in glomerular cyclic GMP content induced by CsA; and (ii) the effect of CsA was not additive with maximal ACh-induced cyclic GMP production which is due to an exclusive activation of the constitutive endothelial NOS (eNOS).

Several mechanisms could explain this CsA-induced activation of the NO pathway. The first possibility is a direct eNOS activation by CsA. This hypothesis was ruled out because incubation of glomeruli from untreated rats with 0.5 mM CsA for 30 min did not alter cyclic GMP content. The second possibility involves the previously described stimulation of endothelin synthesis by CsA (Kon *et al.*, 1990; Perico *et al.*, 1992). In fact, endothelins act through stimulation of two different receptor subtypes: ET_A receptors which are classically responsible for constrictive effects, and ET_B receptors which are non-isopeptide selective receptors of a less definite function since they can induce either vasoconstriction (Seo *et al.*, 1994) or vasodilatation mediated by the NO pathway (Takayanagi *et al.*, 1991). Both ET_A and ET_B receptors are present in the kidney (Terada *et al.*, 1992). Initially, Edwards *et al.* (1992) showed that incubation of rat isolated glomeruli with endothelins induces NO-dependent cyclic GMP production, and then Owada *et al.* (1994) showed that IRL 1620, a specific ET_B agonist, was able to increase glomerular cyclic GMP content through activation of the NO pathway. These results strongly suggested the involvement of ET_B receptors in an endothelin-induced activation of the NO-cyclic GMP cascade. However, the concentrations of endothelins used to stimulate cyclic GMP production in these *in vitro* studies were much higher than those of physiological relevance. Finally, a study by D'Orléans-Juste *et al.* (1994) has demonstrated that the blockade of ET_B receptors with BQ-788, a specific ET_B antagonist, increases the rise in perfusion pressure of rabbit isolated perfused kidneys induced by a concomitant infusion of ET-1; in addition, BQ-788 does not further potentiate the pressor response to ET-1 during L-NAME administration, thus confirming both the involvement of the NO pathway and ET_B activation in this model. This effect was observed with a low concentration of ET-1 (0.5 nM). It may therefore be considered as the first consistent evidence for the physiological role of ET_B-induced renal NO-production. However, these results were obtained by direct administration of endothelins which fits neither physiological nor clinical conditions.

In the present study, the involvement of glomerular endothelins was examined *in vivo* by the use of specific antagonists of ET_A and ET_B receptors. We respectively used BQ-123, a potent and specific ET_A receptor antagonist (Ihara *et al.*, 1992), and BQ-788, a selective ET_B antagonist (Ishikawa *et al.*, 1994). *In vivo* administration of the ET_B antagonist BQ-788 in CsA-treated rats completely abolished the CsA-induced increase in cyclic GMP content, as well as the *in vitro* stimulating effect of 10 nM ET-3 on freshly isolated glomeruli. In contrast, the ET_A antagonist had no effect on baseline glomerular cyclic GMP content or on the stimulating effect of 10 nM ET-3 *in vitro*. These data strongly suggest that activation of the NO pathway after a single injection of CsA results from the *in vivo* activation of ET_B receptors. Two different, but not contradictory, mechanisms could account for this activation. Firstly, CsA could increase endogenous endothelin synthesis in blood

vessels and in the glomeruli themselves. Secondly, CsA could increase the glomerular expression of ET_B receptors. Indeed, Iwasaki *et al.* (1994) have recently demonstrated that CsA is able to increase mRNA expression of ET_B receptors in the renal medulla without any effect on expression in the glomerulus. This observation is consistent with our results since 10 nM ET-3 did not further increase glomerular cyclic GMP content in CsA-treated rats, suggesting that maximum stimulation of a pre-existing population of receptors by increased glomerular synthesis of endothelins occurs rather than increased expression of glomerular ET_B receptors. Moreover, in our experiments, this increase in endothelin synthesis is more likely a consequence of CsA-treatment than of glomerular isolation since (i) immediately after isolation, baseline glomerular cyclic GMP content in CsA-treated rats was significantly higher than in CsA-untreated rats, (ii) the increase in glomerular cyclic GMP content in CsA-treated rats was time-dependent and (iii) baseline glomerular cyclic GMP content remained steady for at least one hour after glomerulus isolation.

The second point of interest is the effects of CsA on the renal constitutive nitric oxide synthase pathway. It is important to emphasize that several findings from *in vivo* or *ex vivo* experiments tended to indicate that CsA is able to inhibit, rather than to stimulate, the constitutive NO pathway because the vasodilator effects of ACh were blunted after CsA (Gerkens, 1989; Takenaka *et al.*, 1992; Diederish *et al.*, 1992). However, this assumption is not supported by the fact that the deleterious effects of NO synthase inhibition with L-NAME on renal haemodynamics are additive with those of CsA (Conger *et al.*, 1994; Bobadilla *et al.*, 1994). Therefore, the possibility that the NO pathway is still operative during CsA treatment cannot be dismissed.

The effects of *in vivo* CsA administration on the expression of eNOS have been poorly investigated. In contrast, it is well established that CsA is able to inhibit the induction of calcium-independent inducible NO synthase (iNOS) by interleukin β in mesangial cells (Mühl *et al.*, 1993). By using RT–PCR to study the relationship between CsA administration and the expression of NO synthases, we failed to detect any effect of CsA on eNOS mRNA expression whereas, as expected, CsA markedly inhibited LPS-induced iNOS mRNA expression.

The present study therefore provides new information concerning the functional status of the constitutive NO pathway during CsA: (i) after a single injection of CsA, the glomerular cyclic GMP content increased briefly and returned to baseline value after 18 h despite a 350 ng ml^{-1} residual CsA blood concentration; (ii) in glomeruli isolated 9 h after CsA administration, incubation with ACh or ET-3 was still able to increase glomerular cyclic GMP content through activation of the eNOS; and (iii) the maximum production of cyclic GMP induced by ACh and ET-3 was slightly but significantly lower (approximately by 20–25%, $P<0.05$) in glomeruli from CsA-treated than in glomeruli from untreated rats.

The initial conclusion is that, the glomerular constitutive NO pathway is transiently activated during acute CsA administration *in vivo* and can be still activated, at least *in vitro*, after glomerular isolation. Although kidneys are an elective target for CsA toxicity, it is suprising that the effects of CsA on tissular cyclic GMP content have been evaluated only on rat aortic rings *in vitro* and that the results obtained were conflicting. Rego *et al.* (1990) showed that incubation of rat aortic rings for 30 min with CsA at high concentration (8.4 mM which is 17 times higher than the concentration in our experiments) elicited a significant decrease in baseline cyclic GMP content. Because such a CsA concentration is also highly detrimental to cultured endothelial cells (Zoja *et al.*, 1986), this decrease in cyclic GMP may have resulted from direct cell toxicity. In the same study, a decrease in aortic ring cyclic GMP content was also observed after 7 days of *in vivo* CsA administration. Unfortunately, the NO-dependence was not established by the use of a NO synthase inhibitor and the possibility that the alteration in tissular cyclic GMP content depended also on atrial natriuretic peptide activation of particulate guanylate cyclase cannot be dismissed (Hamet *et al.*, 1984), since CsA may interfere with sodium homeostasis and, consequently, with atrial natriuretic peptide production (Curtis *et al.*, 1988; Sturrock *et al.*, 1993). In contrast, Gallego *et al.* (1994) detected no change in baseline cylcic GMP content in aortic rings from rats treated for 15 days with CsA 25 mg kg^{-1} day^{-1}(i.m.), but the interval between the last CsA administration and death was not precisely stated. These latter results do not contradict our observations because we observed only a transient increase in glomerular cyclic GMP content and the persistence of this phenomenon during chronic therapy was out of the scope of this study.

The second conclusion is that maximum activation of the constitutive NO pathway, during *in vitro* stimulation with ACh and ET-3, is slightly reduced in CsA-treated rats. It is of interest that the maximum increase of glomerular cyclic GMP content induced by SNP was similar in both treated and untreated groups (Figure 7) indicating no difference in maximum soluble guanylate cyclase activity. This last result ties up with the above-mentioned data concerning reduced cyclic GMP production in aortic rings from CsA treated rats (Rego *et al.*, 1990; Gallego *et al.*, 1994). It is not presently clear why the maximum production of cyclic GMP with ACh and ET-3 during CsA administration was blunted. Several studies on aortic rings (Balligand & Godfraind, 1991) or isolated renal arteries (Diederich *et al.*, 1992) have indicated that the relaxation induced by NO donors remains normal during CsA, suggesting, like our results, that soluble guanylyl cyclase activity is not impaired by CsA and that the limitation of cyclic GMP production results from the direct inhibition of the NO pathway activity. Three main explanations may be discussed to explain the reduced maximum activation of the NO pathway. Firstly, decreased NO synthase expression cannot be excluded, but it is unlikely since our results indicate that eNOS expression is unchanged by CsA. Secondly, CsA could induce a relative decrease in NO synthesis through either an impairment of the enzyme activity or a decrease in substrate availability. In this regard, Dawson *et al.* (1993) suggested that CsA has the ability to prevent *in vitro* dephosporylation of NO synthase and its subsequent activation. In addition, an oral or intraperitoneal supplement with L-arginine (the natural substrate for NO synthases) during CsA treatment has an important protective effect against CsA-induced haemodynamic alterations (De Nicola *et al.*, 1993; Gallego *et al.*, 1993), possibly suggesting a competitive inhibition reversed by an excess of substrate. A third explanation for the reduction in maximum NO pathway activity is a decrease in active NO. Indeed, CsA could reduce NO half-life by increasing superoxide anion production and decreasing the amount of active NO available (Diederich *et al.*, 1994): the decrease glomerular cyclic GMP production induced by ET-3 and ACh stimulation in CsA-treated rats might result from a parallel increase in superoxide anion production.

Our results emphasize the need to examine further ET_B-induced cyclic GMP production during chronic CsA therapy because of its potential advantages. Other recent *in vivo* studies have already suggested that endogenous activation of the NO-pathway during CsA administration should in fact play a protective role (Bobadilla *et al.*, 1994; Amore *et al.*, 1995). Indeed, the selective blockade of ET_A receptors may be beneficial not only by reducing the vasoconstrictor tone but might also favour ET_B receptor stimulation and subsequent cyclic GMP production. Although specific blockade of ET_A receptors has failed to prevent CsA-induced renal structural damage (Hunley *et al.*, 1995) or to affect the renal vasoconstriction induced by ET-1 in rats (Pollock & Opgenorth, 1993), conclusive new insights must be gained from clearing up the precise role of ET_B receptors in the kidney.

In conclusion, we present the first *in vivo* evidence that a therapeutic manoeuvre, i.e. *in vivo* administration of a single

bolus of cyclosporine A, transiently increases cyclic GMP content in *ex vivo* isolated glomeruli through activation of ET_B receptors and subsequent stimulation of the NO pathway. Our results also indicate that CsA does not impair eNOS expression but only slightly reduces NO-dependent glomerular cyclic GMP production. Whether the apparent functional alteration of the constitutive NO synthase pathway is involved in CsA-induced glomerular haemodynamic alterations should be examined further, especially during chronic selective inhibition of endothelin receptor subtypes. Finally, the recently evoked existence of two ET_B receptor subtypes in the kidney (Gellai *et al*., 1996), and their respective involvement as a potential endogenous protective mechanism against CsA nephrotoxicity may be an interesting therapeutic perspective.

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